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Toward Selection of Internalizing Antibodies from Phage Libraries

Baltazar Becerril, Marie-Alix Poul, and James D. Marks[1]

Department of Anesthesia and Department of Pharmaceutical Chemistry, University of California, San Francisco, Room 3C-38, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, California 94110

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Antibodies which bind cell surface receptors in a manner whereby they are endocytosed are useful molecules for the delivery of drugs, toxins, or DNA into the cytosol of mammalian cells for therapeutic applications. Traditionally, internalizing antibodies have been identified by screening hybridomas. For this work, we studied a human scFv (C6.5) which binds ErbB2 to determine the feasibility of directly selecting internalizing antibodies from phage libraries and to identify the most efficient display format. Using wild-type C6.5 scFv displayed monovalently on a phagemid, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor-mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single copies on a phagemid or multiple copies on phage, we define the role of affinity, valency, and display format on phage endocytosis and identify the factors that lead to the greatest enrichment for internalization. Phage displaying bivalent diabodies or multiple copies of scFv were more efficiently endocytosed than phage displaying monomeric scFv and recovery of infectious phage was increased by preincubation of cells with chloroquine. Measurement of phage recovery from within the cytosol as a function of applied phage titer indicates that it is possible to select for endocytosable antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of 10^9. © 1999 Academic Press

Key Words: **endocytosis; ErbB2; gene therapy; phage display; single-chain Fv antibodies; targeted gene delivery.**

Growth factor receptors are frequently overexpressed in human carcinomas and other diseases and thus have been utilized for the development of targeted therapeutics. The *HER2/neu* gene, for example, is amplified in several types of human adenocarcinomas, especially in tumors of the breast and the ovary (1) leading to the overexpression of the corresponding growth factor receptor ErbB2. Targeting of ErbB2 overexpressing cells has been accomplished primarily using anti-ErbB2 antibodies in different formats, including conjugation to liposomes containing chemotherapeutics (2), fusion to DNA carrier proteins delivering a toxic gene (3), and direct fusion to a toxin (4). For many of these targeted approaches, it is necessary to deliver the effector molecule across the cell membrane and into the cytosol. This can be accomplished by taking advantage of normal growth factor receptor biology; growth factor binding causes receptor activation via homo- or heterodimerization, either directly for bivalent ligand or by causing a conformational change in the receptor for monovalent ligand, and receptor mediated endocytosis (5). Antibodies can mimic this process, stimulate endocytosis, become internalized and deliver their payload into the cytosol. In general, this requires a bivalent antibody capable of mediating receptor dimerization (6, 7). In addition, the efficiency with which antibodies mediate internalization differs significantly depending on the epitope recognized (7, 8). Thus for some applications, such as liposomal targeting, only antibodies which bind specific epitopes are rapidly internalized and yield a functional targeting vehicle.

Currently, antibodies which mediate internalization are identified by screening hybridomas. Alternatively, it might be possible to directly select internalizing antibodies from large non-immune phage libraries (9, 10) by recovering infectious phage particles from within cells after receptor mediated endocytosis, as reported for peptide phage libraries (11, 12). Unlike the multivalently displayed peptide phage libraries, however, phage antibody libraries typically display monomeric single chain Fv (scFv) or Fab antibody fragments fused to pIII as single copies on the phage surface using a phagemid system (9, 10). We hypothesized that such monovalent display was unlikely to lead to efficient receptor crosslinking and phage internalization. To de-

[1] To whom correspondence should be addressed. E-mail: jim_marks@quickmail-ucsf.edu.

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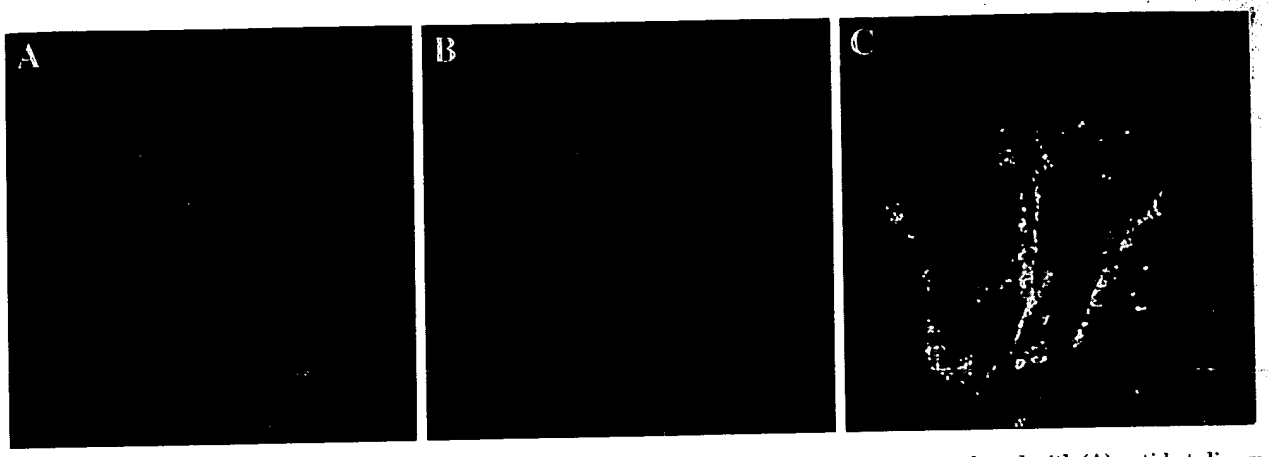

FIG. 1. Internalization of soluble C6.5 scFv and diabody. SKBR3 cells grown on cover slips were incubated with (A) anti-botulinum scFv (B) C6.5 scFv or (C) C6.5 diabody for 2 h at 37°C. Coverslips were washed with PBS and stripping buffer, cells were fixed and permeabilized and intracellular antibodies were detected by confocal microscopy using the anti-myc antibody 9E10, anti-mouse biotinylated antibody and streptavidin Texas-Red.

termine the feasibility of selecting internalizing antibodies and to identify the most efficient display format, we studied a human scFv (C6.5) which binds ErbB2 (13). Using wild type C6.5 scFv, we demonstrate that anti-ErbB2 phage antibodies can undergo receptor mediated endocytosis. Using affinity mutants and dimeric diabodies of C6.5 displayed as either single or multiple copies on the phage surface, we define the role of affinity, valency, and display format on phage endocytosis and identify the factors that lead to the greatest enrichment for internalization. The results indicate that it is possible to select for endocytosable antibodies, even at the low concentrations that would exist for a single phage antibody member in a library of 10^9 members.

MATERIAL AND METHODS

Cells. The SKBR3 breast tumor cell line was obtained from ATCC and grown in RPMI media supplemented with 10% FCS (Hyclone) in 5% CO_2 at 37°C.

Antibodies and antibody phage preparations. The C6.5 scFv phage vector was constructed by subcloning the C6.5 gene as a *SfiI/NotI* fragment from scFv C6.5 pHEN1 (13) into the phage vector fd/*SfiI/NotI* (a gift of Andrew Griffiths, MRC Cambridge, UK). The C6.5 diabody phagemid vector was constructed by subcloning the C6.5 diabody gene (14) as a *NcoI/NotI* fragment into pHEN1 (15). The anti-botulinum scFv phagemid (clone 3D12) (16) C6.5 scFv phagemid (13) and scFv C6ML3-9 scFv phagemid (17) in pHEN1 have been previously described. Phage were prepared (18) from the appropriate vectors and titered on *E. coli* TG1 as previously described (9) using ampicillin (100 μg/ml) resistance for titration of constructs in pHEN1 and tetracyline (50 μg/ml) for titration of constructs in fd. Soluble C6.5 scFv, C6.5 diabody and anti-botulinum scFv were expressed from the vector pUC119mycHis (13) and purified by immobilized metal affinity chromatography as described elsewhere (13).

Detection of internalized native antibody fragments and phage antibodies. SKBR3 cells were grown on coverslips in 6-well culture plates (Falcon) to 50% of confluency. Culture medium was renewed 2 h prior to the addition of 5×10^{11} cfu/ml of phage preparation (the phage preparation representing a maximum of 1/10 of the culture medium volume) or 20 μg/ml of purified scFv or diabody in phosphate buffered saline, pH 7.4 (PBS). After 2 h of incubation at 37°C, the wells were quickly washed 6 times with ice cold PBS and 3 times for 10 min each with 4 mL of stripping buffer (50 mM glycine pH 2.8, 0.5 M NaCl, 2M urea, 2% polyvinylpyrrolidone) at RT. After 2 additional PBS washes, the cells were fixed in 4% paraformaldehyde (10 min at RT), washed with PBS, permeabilized with acetone at −20°C (30 s) and washed again with PBS. The coverslips were saturated with PBS–1% BSA (20 min. at RT). Phage particles were detected with biotinylated anti-M13 immunoglobulins (5 Prime-3 Prime, Inc, diluted 300 times) (45 min at RT) and Texas red-conjugated streptavidin (Amersham, diluted 300 times) (20 min. at RT). Soluble scFv and diabodies containing a C-terminal myc peptide tag were detected with the mouse mAb 9E10 (Santa Cruz Biotech, diluted 100 times) (45 min. at RT), anti-mouse biotinylated immunoglobulins (Amersham, diluted 100 times) and Texas red-conjugated streptavidin. Optical confocal sections were taken using a Bio-Rad MRC 1024 scanning laser confocal microscope. Alternatively, slides were analyzed with a Zeiss Axioskop UV fluorescent microscope.

Recovery and titration of cell surface bound or internalized phage. Subconfluent SKBR3 cells were grown in 6-well plates. Culture medium was renewed 2 h prior to the experiment. Cells were incubated for varying times with different concentrations of phage preparation at 37°C (specific details for each experiment are provided in the table or figure legends). Following PBS and stripping buffer washes, performed exactly as described above for detection of internalized native antibody fragments and phage antibodies, the cells were washed again twice with PBS and lysed with 1 mL of 100 mM triethylamine (TEA). The stripping buffer washes and the TEA lysate were neutralized with 1/2 volume of Tris–HCl 1M, pH 7.4. For some experiments (see figure legends for specifics), cells were trypsinized after the three stripping buffer washes, collected in a 15-ml Falcon tube, washed twice with PBS and then lysed with TEA. In experiments performed in the presence of chloroquine, SKBR3 cells were preincubated for 2 h in the presence of complete medium containing 50 μM chloroquine prior to the addition of phage. Corresponding control samples in the absence of chloroquine were prepared at the same time. For all experiments, phage were titered on *E. coli* TG1 as described above.

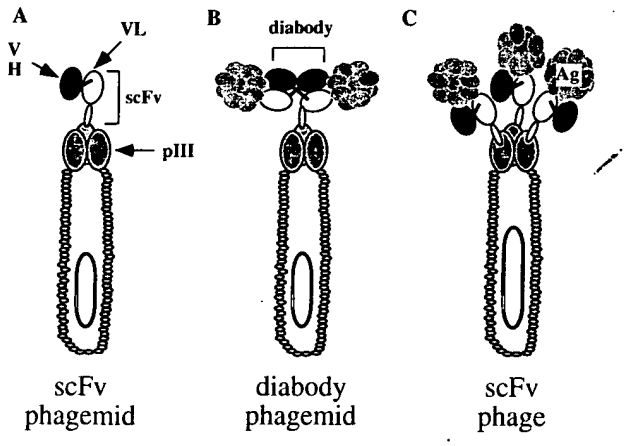

FIG. 2. Antibody phage display. Cartoon of phage displaying (A) a single scFv (B) a single diabody or (C) multiple scFv. scFv, single chain Fv antibody fragment; V_H, Ig heavy chain variable domain; V_L, Ig light chain variable domain; pIII, phage minor coat protein pIII; Ag, antigen bound by scFv.

RESULTS

1. The Model System Utilized to Study Phage Antibody Internalization

The human anti-ErbB2 scFv C6.5 was obtained by selecting a human scFv phage antibody library on recombinant ErbB2 extracellular domain (13). C6.5 scFv binds ErbB2 with a $K_d = 1.6 \times 10^{-8}$ M and is a stable monomeric scFv in solution with no tendency to spontaneously dimerize or aggregate (13). To determine the impact of affinity on internalization, we studied a scFv (C6ML3-9) which differs from C6.5 by 3 amino acids (17). C6ML3-9 scFv is also a stable monomer in solution and binds the same epitope as C6.5 scFv but with a 16-fold lower K_d (1.0×10^{-9} M) (17, 19). Since receptor homodimerization appears to typically be requisite for antibody internalization we also studied the dimeric C6.5 diabody (14). Diabodies are scFv dimers where each chain consists of heavy (V_H) and light (V_L) chain variable domains connected using a peptide linker which is too short to permit pairing between domains on the same chain. Consequently, pairing occurs between complementary domains of two different chains, creating a stable noncovalent dimer with two binding sites (20). The C6.5 diabody was constructed by shortening the peptide linker between the Ig V_H and V_L domains from 15 to 5 amino acids and binds ErbB2 on SKBR3 cells bivalently with a K_d approximately 40-fold lower than C6.5 (4.0×10^{-10} M) (14).

Native C6.5 scFv and C6.5 diabody was expressed and purified from *E. coli* and analyzed for endocytosis into ErbB2 expressing SKBR3 breast tumor cells by immunofluorescent confocal microscopy. As expected, monomeric C6.5 scFv is not significantly internalized whereas the dimeric C6.5 diabody can be detected in the cytoplasm of all cells visualized (Fig. 1).

For subsequent experiments, the C6.5 and C6ML3-9 scFv and C6.5 diabody genes were subcloned for expression as pIII fusions in the phagemid pHEN-1 (15). This should yield phagemid predominantly expressing a single scFv or diabody-pIII fusion after rescue with helper phage (21) (Figs. 2A and 2B). Diabody phagemid display a bivalent antibody fragment resulting from intermolecular pairing of one scFv-pIII fusion molecule and one native scFv molecule (Fig. 2B). The C6.5 scFv gene was also subcloned into the phage vector fd-Sfi/Not. This results in phage with 3 to 5 copies each of scFv-pIII fusion protein (Fig. 2C). The human breast cancer cell line SKBR3 was used as a target cell line for endocytosis. Its surface ErbB2 density is approximately 1.0×10^6 per cell (22).

2. C6.5 Phagemids Are Endocytosed by Human Cells

C6.5 scFv phagemids were incubated for 2 h with SKBR3 cells grown on coverslips at 37°C to allow active internalization. Cells were extensively washed with PBS to remove non specific binding and washed an additional three times with high salt and low pH (stripping) buffer to remove phage specifically bound to cell surface receptors. Internalized phagemid were de-

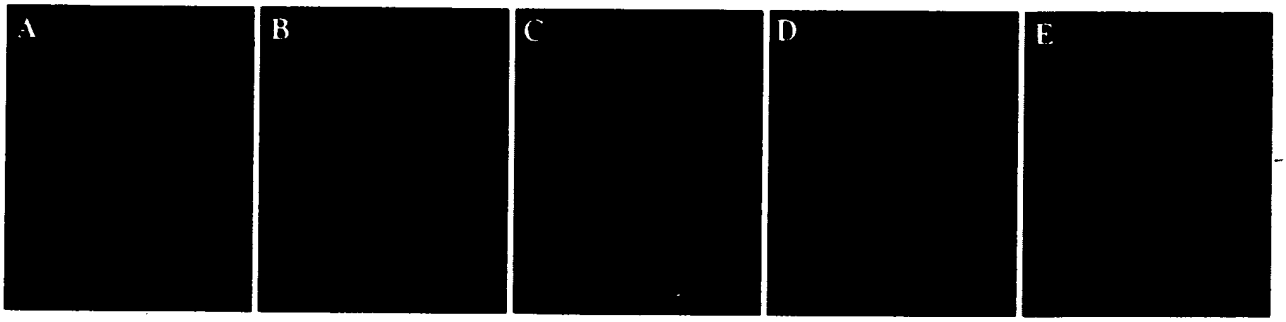

FIG. 3. Internalization of C6.5 phage derivatives. SKBR3 cells grown on coverslips were incubated with 5.0×10^{11} cfu/ml (A) anti-botulinum phagemid (B) scFv C6.5 phagemid (C) C6ML3-9 phagemid (D) C6.5 diabody phagemid or (E) C6.5 phage for 2 h at 37°C. The cells were treated as described in the legend to Fig. 1 and intracellular phage were detected with a fluorescent microscope using biotinylated anti-M13 antiserum and Texas-Red streptavidin.

TABLE 1

Titration of Membrane-Bound and Intracellular Phage

Phage antibody	Cell surface phage titer ($\times 10^{-5}$)			Intracellular phage titer ($\times 10^{-5}$)
	1st wash	2nd wash	3rd wash	
Anti-botulinum phagemid	280	36	2.8	15
C6.5 scFv phagemid	600	96	7.6	52
C6ML3-9 scFv phagemid	2500	140	32	270
C6-5 diabody phagemid	1800	120	13	450
C6.5 scFv phage	2300	620	56	2200

Note. 3.0×10^{11} cfu of monovalent C6.5 scFv phagemid, 16-fold higher affinity monovalent C6ML3-9 scFv phagemid, bivalent C6.5 diabody phagemid, or multivalent C6.5 fd phage were incubated with subconfluent SKBR3 cells for 2 h at 37°C. Cells were washed 6 times with PBS, 3 times with stripping buffer, and then lysed to recover intracellular phage. The various fractions were neutralized and the phage titered. The total number of cfu of each fraction is reported. Nonspecific anti-botulinum phagemid were used to determine nonspecific recovery.

tected with a biotinylated M13 antiserum recognizing the major coat phage protein pVIII. An anti-botulinum toxin phagemid was used as a negative control. Staining was analyzed by using immunofluorescent microscopy (Fig. 3). Approximately 1% of the cells incubated with C6.5 scFv phagemid showed a strong intracellular staining consistent with endosomal localization (Fig. 3B) while no staining was observed for anti-botulinum phagemid (Fig. 3A). Furthermore, no staining was seen if the incubation was performed for 2 h at 4°C instead of 37°C (data not shown). Staining performed after the PBS washes but before washing with stripping buffer showed membrane staining of all the cells, indicating that multiple washes with stripping buffer is necessary to remove surface-bound phagemids. The results also indicate that only a fraction of the cell bound phage are endocytosed.

3. Increased Affinity and Bivalency Lead to Increased Phage Endocytosis

We compared the internalization of C6.5 scFv, C6ML3-9 scFv and C6.5 diabody phagemid and C6.5 scFv phage using immunofluorescence. Both C6ML3-9 scFv and C6.5 diabody phagemid as well as C6.5 scFv phage yielded increased intensity of immunofluorescence observed at the cell surface compared to C6.5 scFv phagemid. For C6ML3-9 scFv phagemid, approximately 10% of the cells showed intracellular fluorescence after 2 h of incubation (Fig. 3C). This value increased to approximately 30% of cells for the dimeric C6.5 diabody phagemid (Fig. 3D) and 100% of cells for multivalent C6.5 scFv phage (Fig. 3E).

4. Infectious Phage Can be Recovered from within the Cell and Their Titer Correlates with the Level of Uptake Observed Using Immunofluorescence

To determine if infectious phage antibody particles could be recovered from within the cell, we incubated approximately 5.0×10^5 SKBR-3 cells for 2 h at 37°C with 3.0×10^{11} cfu of the different phagemid or phage. Six PBS washes were used to remove non-specifically bound phage and specifically bound phage were removed from the cell surface by three consecutive washes with stripping buffer (washes I, II, and III respectively, Table 1). The cells were then lysed with 1 mL of a 100 mM triethylamine solution (TEA) (representing the intracellular phage). The three stripping washes and the cell lysate were neutralized and their phage titer was determined by infection of *E. coli* TG1. The titers of phage recovery are reported in Table 1.

Considerable background binding was observed in the first stripping wash for the anti-botulinum phage even after 6 PBS washes (2.8×10^7 cfu, Table 1). This value likely represents phage non-specifically bound to the cell surface as well as phage trapped in the extracellular matrix. The amount of surface bound phage increased only 2.1-fold above this background for C6.5 scFv phagemid (Tables 1 and 2). With increasing affinity and avidity of the displayed C6.5 antibody fragment, the titer of cell surface bound phagemid or phage increased (Table 1). The titer of phage in the consecutive stripping washes decreased approximately 10-fold with each wash. These additional stripping washes led to a minor increase in the titer of specific phage eluted compared to the background binding of the anti-botulinum phage (2.7-fold for C6.5 scFv phagemid to 20-fold for C6.5 scFv phage, Table 2). The only exception was the titer of the C6.5 diabody phagemid, where the ratio actually decreased from 6.4- to 4.6-fold. This is likely due to the fact that in the diabody the V_H and V_L domains that comprise a single binding site are not covalently attached to each other via the peptide linker. This increases the likelihood that a stringent eluent (like glycine) could dissociate V_H from V_L and abrogate binding to antigen.

Three stripping washes were required to ensure that the titer of phage recovered after cell lysis was greater

TABLE 2

Specific Enrichment of Anti-ErbB2 Phage Compared to Anti-Botulinum Phage

Phage antibody	Anti-ErbB2/anti-botulinum phage titer ratio[a]			Intracellular/cell surface phage ratio[b]
	Cell surface (1st wash)	Cell surface (3rd wash)	Intracellular	
C6.5 scFv phagemid	2.14	2.7	3.5	6.8
C6ML3-9 scFv phagemid	8.9	11.4	18	8.4
C6.5 diabody phagemid	6.4	4.6	30	35
C6.5 scFv phage	8.2	20	146	39

[a] The titers of anti-ErbB2 phage are divided by the titers of the anti-botulinum phage (Table 1) to derive an enrichment ratio for specific vs nonspecific binding or internalization.

[b] The titer of intracellular phage is divided by the titer of cell surface bound phage (Table 1) to derive the ratio of internalized phage vs surface bound phage.

than the titer in the last stripping wash (Table 1). We presumed that after three stripping washes, the majority of the phage eluted represented infectious particles from within the cell rather than from the cell surface. In fact, since the cell lysate titer observed with non-specific anti-botulinum phage was considerable (1.5×10^6) and greater than observed in the last stripping wash, it is likely that many phage remain trapped within the extracellular matrix and relatively inaccessible to the stripping buffer washes. Some anti-botulinum phage might also be non-specifically endocytosed by cells, but this is likely to be a small amount given the immunofluorescence results (Fig. 3). The titer of phage in the TEA fraction increased with increasing affinity and avidity of C6.5, with the highest titers observed for the dimeric C6.5 diabody phagemid and the multivalent C6.5 scFv phage (Table 1). The values represent a 30-fold (C6.5 diabody phagemid) and 146-fold (C6.5 scFv phage) increase in titer compared to the anti-botulinum phage (Table 1). We have presumed that the increase in the phage titer in the cell lysate compared to the last stripping wash is due to endocytosed phage. In fact, some of these phage could have come from the cell surface or intracellular matrix. While this could be true for a fraction of the phage from the cell lysate, the immunofluorescence results indicate that at least some of the phage are endocytosed. One indicator of the relative fraction of endocytosed phage for the different C6.5 molecules is to compare the amount of phage remaining on the cell surface prior to cell lysis (last stripping wash) with the amount recovered after cell lysis. This ratio shows only a minor increase for monovalent C6.5 scFv or C6ML3-9 scFv phagemid (6.8- and 8.4-fold, respectively) compared to anti-botulinum phagemid (5.4) (Table 2). In contrast the ratios for dimeric C6.5 diabody phagemid and multivalent C6.5 scFv phage increase to a greater extent (35 and 39, respectively) compared to anti-botulinum phagemid.

5. *Increasing the Enrichment Ratios of Specifically Endocytosed Phage*

The results above indicate that phage antibodies can undergo receptor mediated endocytosis and remain infectious in a cell lysate. Selection of internalized phages from a phage library requires the optimization of the method to increase enrichment of specifically internalized phages over non-internalized phage. Two parameters can be improved: (i) reduction of the recovery of non-specific or non-internalized phage and (ii) preservation of the infectivity of internalized phage. To examine these parameters, we studied wild-type C6.5 scFv phagemid. We chose this molecule because it was clearly endocytosed based on confocal microscopy, yet was the molecule undergoing the least degree of specific endocytosis. C6.5 scFv phagemid also represents the most commonly utilized format for display of non-immune phage antibody libraries (single copy pIII in a phagemid vector) and has an affinity (16 nM) more typical of K_d's of scFv from such libraries than the affinity matured C6ML3-9 scFv (10, 23).

a. Reducing the background of non-internalized phage. To reduce the background of nonspecific phage recovery, we studied the effect of trypsinizing the cells prior to TEA lysis. This should remove phage trapped in the extracellular matrix. Trypsinization also dissociates the cells from the cell culture flask, permitting transfer to a new vessel and elimination of any phage bound to the cell culture flask. For these experiments, C6.5 scFv phagemid (5.0×10^8 ampicillin resistant cfu) were mixed with a 1000-fold excess of wild type fd phage (5.0×10^{11} tetracycline-resistant cfu). After incubation of phagemid with SKBR-3 cells for 2 h at 37°C, cells were washed with PBS and three times with stripping buffer. Cells were then directly lysed with TEA or treated with trypsin, washed twice with PBS and then lysed with TEA. Phagemid in the first stripping wash and the cell lysate were titered by infection of *E. coli* TG1 and plated on ampicillin and tetracycline

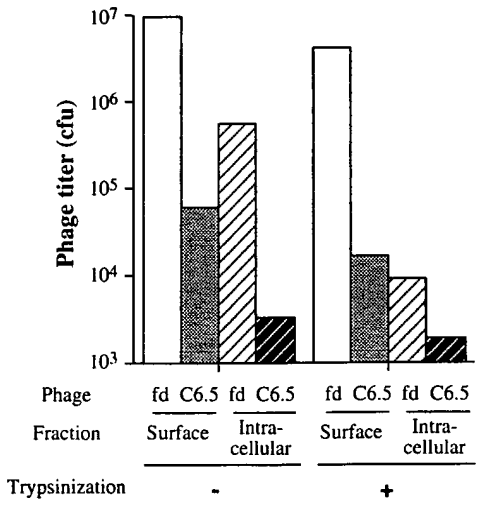

FIG. 4. Effect of trypsinization on the enrichment of antigen specific phage. A mixture of fd phage (5.0×10^{11} cfu) and C6.5 scFv phagemid (5.0×10^8 cfu) was incubated with SKBR3 cells for 2 h at 37°C. Washes were performed either as described in Table 1 (−) or cells were trypsinized prior to cell lysis (+). Phage present in the first stripping buffer wash (cell surface phage) and the cell lysate (intracellular phage) were titered in the presence of ampicillin (C6.5 phagemid) or tetracycline (fd phage).

plates. The titer of fd phage and C6.5 scFv phagemid recovered from the cell surface was comparable for the two experimental groups (Fig. 4). The ratio of fd phage/C6.5 scFv phagemid in the cell surface fractions (160/1 and 250/1) yields a 4- to 6-fold enrichment achieved by specific cell surface binding from the initial 1000-fold ratio. Without trypsinization, the ratio of fd phage/C6.5 scFv phagemid in the cell lysate increases only 6.1-fold; in contrast, the ratio increases 209-fold with trypsinization (Fig. 4). This results from a 60-fold reduction in nonspecific binding with only a minor reduction in the amount of specific phage recovery (Fig. 4).

b. Improving the recovery of infectious internalized phage. To increase the recovery of infectious internalized phage, we studied whether prevention of lysosomal acidification through the use of chloroquine would protect endocytosed phages from endosomal degradation (12). SKBR3 cells were incubated with chloroquine and either C6.5 scFv phagemid or anti-botulinum phagemid. Cell lysates were titered at various time points to determine the number of intracellular phagemid. C6.5 scFv phagemid were present at the 20-min time point and the amount of phagemid was comparable with or without the addition of chloroquine. At later time points, approximately twice as much infectious phagemid was recovered with the use of chloroquine. In contrast, much lower amounts of anti-botulinum phage were present and chloroquine had no effect on the titer, suggesting that the phagemid result from non-specific surface binding rather than non-specific endocytosis into endosomes. Overall, the results indicate that prevention of lysosomal acidification increases the amount of infectious phage recovered for incubations longer than 20 min (Fig. 5).

6. Recovery of Internalized Phage at Low Phage Concentrations

Only very large phage antibody libraries containing more than 5.0×10^9 members are capable of generating panels of high affinity antibodies to all antigens (10, 23, 24). Since phage can only be concentrated to approximately 10^{13} cfu/ml, a typical phage preparation from a large library will only contain 10^4 copies of each member. Thus selection of libraries for endocytosis could only work if phage can be recovered when applied to cells at titers as low as 10^4. We therefore determined the recovery of infectious phage from within SKBR3 cells as a function of the phage titer applied. SKBR3 cells were incubated with C6.5 scFv, C6ML3-9 scFv or C6.5 diabody phagemids or C6.5 scFv phage for 2 h at 37°C. Cells were washed three times with stripping buffer, trypsinized and washed twice with PBS. Cells were lysed and intracellular phage titered on *E. coli* TG1. Phage recovery increased with increasing phage titer for all phage studied (Fig. 6). For monovalently displayed antibodies, phagemid could not be recovered from within the cell at input titers less than 3.0×10^5 (C6.5 scFv) to 3.0×10^6 (C6ML3-9 scFv) This threshold decreased for bivalent and multivalent display (3.0×10^4 for C6.5 diabody phagemid and C6.5 scFv phage).

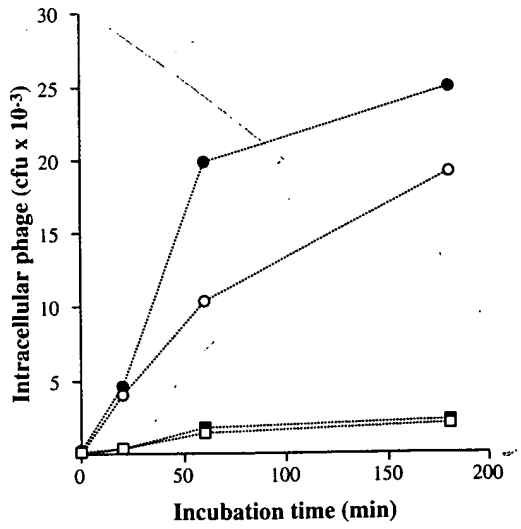

FIG. 5. Effect of incubation time and chloroquine on the recovery of antigen specific phage. SKBR3 cells were incubated in the presence (■,) or absence (□, ○) of chloroquine (50 μM) for 2 h prior to the addition of anti-botulinum phagemid (□, ■) or C6.5 scFv phagemid (○,) (1.5×10^9 cfu/ml). Cell samples were taken at 0 min, 20 min, 1 h, or 3 h after phage addition, washed as described in the legend to Fig. 4 including the trypsinization step and intracellular phages titered.

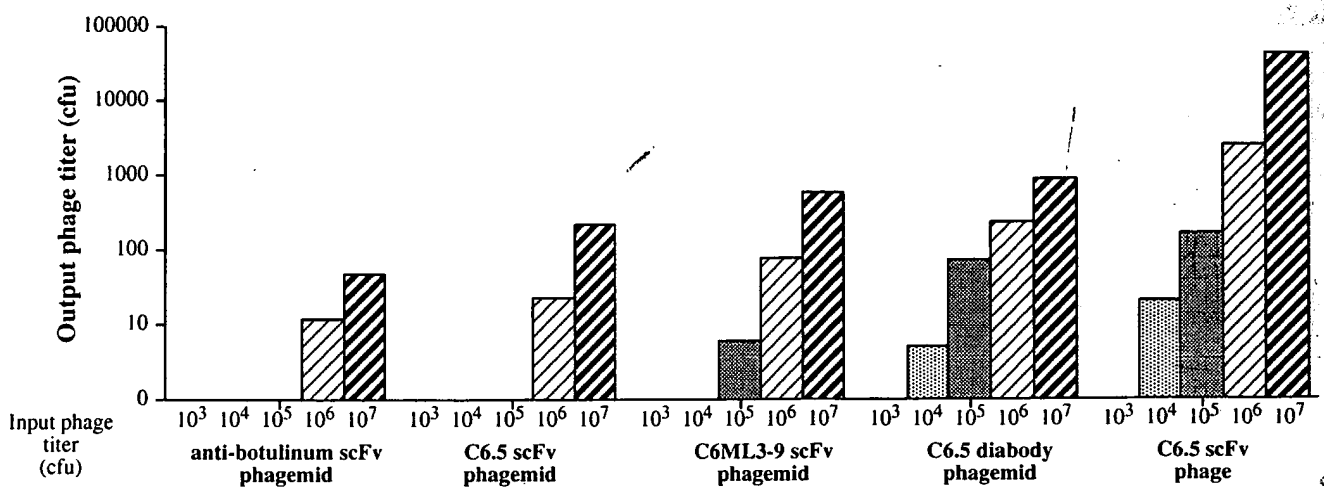

FIG. 6. Effect of phage concentration on the recovery of intracellular phage. Various concentrations of C6.5 scFv phagemid, C6ML3-9 scFv phagemid, C6.5 diabody phagemid or C6.5 scFv phage (input phage titer) were incubated with subconfluent SKBR3 cells grown in 6-well plates for 2 h at 37°C. Cells were treated as described in the legend to Fig. 4 including the trypsinization step and intracellular phage were titered (output phage titer).

DISCUSSION

We demonstrate for the first time that phage displaying an anti-receptor antibody can be specifically endocytosed by receptor expressing cells and can be recovered from the cytosol in infectious form. The results demonstrate the feasibility of directly selecting internalizing antibodies from large non-immune phage libraries and identify the factors that will lead to successful selections. Phage displaying anti-ErbB2 antibody fragments are specifically endocytosed by ErbB2 expressing SKBR3 cells, can be visualized within the cytosol and can be recovered in an infectious form from within the cell. When monovalent scFv antibody fragments were displayed monovalently in a phagemid system, recovery of internalized phage was only 3.5- to 18-fold above background. Display of bivalent diabody or multivalent display of scFv in a phage vector increased recovery of internalized phage to 30- to 146-fold above background. This result is consistent with our studies of native monomeric C6.5 scFv and dimeric C6.5 diabody as well as studies of other monoclonal anti-ErbB2 antibodies where dimeric IgG but not monomeric Fab dimerize and activate the receptor and undergo endocytosis (7, 8). In fact it is likely that endocytosis of C6.5 and C6ML3-9 scFv phagemids reflect the small percentage of phage displaying two or more scFv (21). The importance of valency in mediating either high avidity binding or receptor crosslinking and subsequent endocytosis is confirmed by the only other report demonstrating specific phage endocytosis. Phage displaying approximately 300 copies of a high affinity Arg-Gly-Asp integrin binding peptide on pVIII were efficiently endocytosed by mammalian cells (11). Recovery of phage after endocytosis also increases the specificity of cell selections compared to recovery of phage from the cell surface. Thus enrichment ratios for specific vs nonspecific surface binding range from 2- to 20-fold. These values are comparable to the approximately 10-fold enrichment reported by others for a single round of cell surface selection (25, 26). In contrast our enrichment ratios for specific vs non-specific endocytosis range from 3.5- to 146-fold.

Based on these results, selection of internalizing antibodies from phage antibody libraries would be most successful with either homodimeric diabodies in a phagemid vector or multivalent scFv using a phage vector. While no such libraries have been published, there are no technical barriers preventing their construction. Multivalent libraries would present the antibody fragment in the form most likely to crosslink receptor and undergo endocytosis. Antibodies from such libraries would need to be bivalent to mediate endocytosis. Alternatively, monomeric receptor ligands can activate receptors and undergo endocytosis, either by causing a conformational change in the receptor favoring the dimeric form or by simultaneously binding two receptors. Monomeric scFv that bound receptor in a similar manner could also be endocytosed. Thus selection of libraries of monovalent scFv in a phagemid vector could result in the selection of ligand mimetics that activate receptors and are endocytosed as monomers. Such scFv could be especially useful for the construction of fusion molecules for the delivery of drugs, toxins or DNA into the cytoplasm. Since antibodies which mediate receptor internalization can cause receptor downregulation and growth inhibition (8, 27–29), selection for endocytosable antibodies may also identify antibodies which directly inhibit or modulate cell growth.

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